Physiological up-regulation of inhibitory receptors FcγRII and CR1 on memory B cells is lacking in SLE patients

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Abstract

Under physiological conditions immune complexes (IC) are efficiently cleared from the circulation and meanwhile provide important feedback signals for the immune system via FcγRs and complement receptors. Dysregulation of these mechanisms have been implicated in conditions where IC concentrations reach pathological levels and inflict diseases, like systemic lupus erythematosus (SLE). Our aim was to compare distinct sub-populations of CD19+ B cells of healthy individuals and SLE patients with regard to their expression of FcγRII, CD35 and complement receptor type 2 (CR2, CD21) and sIgG/IgM. The following four groups of peripheral CD19+ B cells were investigated: IgM+/CD27+ naive, IgM+/CD27− and IgM−/CD27+ memory cells and CD27high plasmablasts. We demonstrate that the expression of the inhibitory receptors FcγRII and CR1 is up-regulated on peripheral memory B cells of healthy controls, whereas this up-regulation is considerably impaired on the memory B cells of SLE patients. This reduction affects both the IgM+ and switched memory B cells. We found a striking difference between the expression of complement receptors CD21 and CD35; namely, no up-regulation of CD21 occurred on the memory B cells of healthy donors, and its decreased expression in SLE patients was characteristic for both the CD27− naive and the CD27+ memory B-cell populations. Our results clearly demonstrate that the previously reported reduced expression of IC-binding receptors is mainly due to the disturbed memory compartment; however, the higher frequency of CD19+/CD27high/sIglow plasmablasts expressing minimal levels of these receptors also contributes to this diminution.

Introduction

The importance of complement receptor type 1 (CR1, CD35) and complement receptor type 2 (CR2, CD21) along with FcγRs (FcγRI, FcγRII and FcγRIII) has been demonstrated in several autoimmune animal models (1, 2). Both Fc gamma and complement receptors are involved in the binding of immune complexes (IC) and delivering signals, which influence effector cell functions and the fate of autoreactive B cells. For the generation and control of an appropriate humoral immune response, balanced signalling through these receptors has an elemental role. FcRs and the complement system, as part of the innate immune system, are involved in early immune defence and subsequently activate and instruct the adaptive immune system in an interconnected way. Coordinated activation and signalling through these receptors are necessary to avoid uncontrolled inflammation; thus alterations in their expression and/or function are potential susceptibility factors in the pathogenesis of IC-mediated autoimmune diseases.

Autoimmune diseases are often characterized by the appearance of elevated levels of self-reactive antibodies indicating disturbances in the B-cell compartment. Decreased FcγR type II (FcγRII), CR1 and CR2 expression may accompany abnormal B-cell functions and has been reported in human autoimmune disease such as systemic lupus erythematosus (SLE) and rheumatoid arthritis. The first studies investigating complement receptor expression in patients with
SLE showed that erythrocytes bear low levels of CR1 (3, 4). Later on, the observations were extended to blood leukocytes, and besides the reduced expression of CR1 on neutrophils and B cells (5), the expression of CR2 also proved to be decreased on B cells in patients suffering from SLE (6). However, no relationship between CR1 expression on B lymphocytes and the severity of the disease was found. It has also been reported that CR2 expression is strongly reduced on synovial B cells of arthritis patients compared with their peripheral B cells (7). Thus, both local and systemic inflammatory conditions are associated with the down-modulation of CR1 and CR2 on B lymphocytes. Of the human complement receptors, only CR1 exhibits molecular weight polymorphism and variability in its cell-surface density. This density polymorphism of human CR1 is, however, characteristic only for erythrocytes and does not affect B cells (8).

Previous studies on FcγRIIb of B cells from patients with SLE have focused on the presence of specific promoter polymorphisms influencing surface levels of the receptor. Polymorphisms affecting the transmembrane domain (9, 10) and the promoter region (11) have been identified in the human FcγRIIb gene, which was associated to human SLE. These polymorphisms lead to decreased transcription and surface expression of the inhibitory FcγRIIb on activated B cells, moreover to the inability to associate with lipid rafts. Although studies in animal models have suggested that genetically determined abnormalities in these IC-binding receptors may have a causative role in autoimmune disorders, one should interpret and apply these results to humans carefully. The pathogenic potential of inhibitory FcγRs and complement receptors in human autoimmune disorders is still controversial. Whether reduced receptor expression represents an inherited genetic phenomenon or is a secondary event caused by complement containing IC or antibodies directed against these receptors is unclear. The functional consequences of these reductions are also not known and the question which B-cell sub-populations are affected is also important to answer. Distribution of these receptors on distinct B-cell sub-populations has not been investigated so far, not even in healthy individuals.

In this study, we investigated the expression of FcγRII (CD32), CR1 (CD35) and CR2 (CD21) and membrane Ig on distinct peripheral B-cell sub-populations of healthy individuals and SLE patients. Peripheral CD19+ B cells were divided into four groups based on levels of CD27 expression as a typical marker for memory B cells: IgM+/CD27− naive, IgM+/CD27+ and IgM−/CD27+ memory cells, as well as CD27high plasmablasts. We demonstrate that the previously reported reduction of Fc and complement receptors is partly due to the disturbed memory compartment and partly to the higher frequency of CD19+/CD27high plasmablasts expressing minimal levels of these receptors.

Methods

Patients and controls

All patients satisfied the criteria for a definitive diagnosis of SLE (12). Disease activity was assessed by the SLE disease activity index score (13). For the studies, SLE patients showing similar receptor expression pattern on their peripheral B cells were selected. As controls, age- and sex-matched healthy subjects were included. The study was approved by the local ethical committee (Institutional Review Board of National Institute of Rheumatology and Physiotherapy) and written informed consent was obtained from each participant.

Flow cytometry

Immunofluorescence measurements were performed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA, USA) and the WinMDI 2.8 software. PBMCs were isolated from heparinized blood by Ficoll Hypaque (Amersham Bioscience) density gradient centrifugation and washed twice in PBS. On the basis of forward- and side-scattering properties, lymphocytes were counted and dead cells were excluded. B cells were stained with the following conjugated monoclonal antibodies: anti-CD35–FITC (Southern Biotechnology), anti-CD21–FITC (Dako), anti-CD32–FITC (clone FL18.26, BectonDickinson), anti-CD27–PE (Caltag Laboratories), anti-IgG–Cy5 and anti-IgM–Cy5 F(ab′)2 (Jackson ImmunoResearch). Cytofluorimetric analysis of distinct B-cell sub-populations was performed on CD19-gated lymphocytes. CD19 was detected by biotin-labelled anti-CD19 (BectonDickinson) and SA-PerCP–Cy5.5 (PharMingen). Ig isotype-matched FITC- and PE-conjugated mouse antibodies were used as negative controls for non-specific staining. A total of 3 × 10⁵ PBMCs were washed in PBS and stained using the appropriate antibodies according to the manufacturer’s instruction. After incubation on ice for 30 min, cells were washed and re-suspended in 200 μl PBS containing 1% FCS and 0.15 NaN₃. Data of 80 000 cells were collected.

Statistical analysis

Data are expressed as mean ± SE and statistical significance was determined using the Mann–Whitney U-test. Data were analysed by Prism software, version 4.0 (GraphPad Software). Results were considered statistically significant if P < 0.05.

Results

IgM+ and IgM− memory cells from SLE patients fail to up-regulate the inhibitory FcγRII

It is known that FcγRII expression on B cells of SLE patients is strongly reduced as compared with healthy individuals (14, 15). Since it has been shown that FcγRII expression on B cells is changing during their maturation, in further studies we aimed to clarify which B-cell sub-populations are affected. To this end, the following four groups of CD19+ peripheral B cells were analysed: IgM+/CD27− naive, IgM−/CD27+ and IgM−/CD27+ memory cells and CD27high plasmablasts. As shown in Fig. 1, memory cells of healthy individuals displayed an increased level of FcγRII expression when compared with naive cells (Fig. 1A and C), while memory B cells from SLE patients failed to up-regulate this receptor (Fig. 1B and C). It is also demonstrated that CD27+/IgM+ cells from healthy individuals express more FcγRII than IgM− memory B cells (Fig. 1A and C). Reduction in FcγRII expression found in SLE affected both IgM+ and switched memory cells (Fig. 1C). Interestingly, the distribution of FcγRII-expressing cells among the IgM+ memory cells was different from the switched memory cells in the case of
healthy donors, being the range of positive lymphocytes much wider in the latter case (Fig. 1A). In SLE, there was no major difference in this respect (Fig. 1B).

As seen in Fig. 1(C), both IgM+ and IgM− memory B cells derived from the autoimmune patients had significantly lower surface levels of FcγRII than the healthy controls (Fig. 1C), most probably due to a failure to up-regulate FcγRII expression during differentiation to memory cells.

Although the antibody we used cannot distinguish the activating FcγRIIa and the inhibitory FcγRIIb isoforms, we found that the mRNA level of FcγRIIa in peripheral B cells was near or below the limit of detection (data not shown); hence, the inhibitory IIb isoform dominates in these samples.

Different expression pattern of CR1 and CR2 on naive and memory B cells of healthy donors and SLE patients

In studies using lupus-prone MRL/lpr mice, it had been shown that the level of complement receptors CR1/2 is strongly reduced on B lymphocytes (16), and the decrease could be detected before nephritis and elevated levels of auto-antibodies appeared. While in the mouse CR1 and CR2 are the alternatively spliced products of a common gene, in humans these two receptors are encoded by distinct genes. This dissimilarity is reflected in different functions exerted by the human receptors CR1 and CR2 (17, 18). As the expression level of human CR1 and CR2 on distinct B-cell sub-populations has not been defined so far, we set out to investigate it on B cells of healthy individuals and SLE patients, as well.

As shown in Fig. 2(A and C), the level of CR1 was found significantly higher on memory B cells in healthy donors, similarly to the expression of FcγRII, but there was no difference between the IgM+ and switched memory compartment. In contrast to FcγRII and CR1, however, the expression of CR2 was not different on naive and memory cells and no up-regulation could be seen in the control group (Fig. 3A). These findings point to further differences regarding the expression and regulation of human CR1 and CR2.

Investigating CR1 and CR2 expression on CD19+ B cells derived from patients with SLE, a significant decrease of both receptors was found, however, different sub-populations were affected. While a strongly reduced CR1 level could be measured only on memory cells (Fig. 2C), a lower CR2...
expression was characteristic for both the naive and memory cells (Fig. 3C). Regarding CR1 expression, both IgM+ and switched memory cells were affected (Fig. 2C), similarly to our finding with FcεRII.

IgM+ and IgG+ memory cells of SLE patients express lower surface Ig levels than B cells of healthy donors

Comparing surface Ig (slg) expression by CD27−/IgM− naive and CD27+/IgM− memory B lymphocytes from healthy individuals, a significantly higher slgM level could be detected in the case of the latter (Table 1). The difference between naive and memory B cells, however, was not observed in the SLE group (Table 1). IgM+ naive B cells of SLE patients expressed higher slgM levels than those of the healthy donors (Table 1). Since naive and transitional B cells present in the CD27− compartment were not investigated separately, a higher frequency of transitional B cells expressing higher slgM levels in SLE may lie behind this phenomenon. Investigating slg expression, we found a decrease on both IgM+ and IgG+ memory B cells of SLE patients as compared with healthy controls (Table 1).

CD19+/CD27high/slglow plasmablasts of healthy donors and SLE patients express minimal levels of FcγRII and CR1

As previously reported, patients with SLE have a disturbed ratio of naive and memory B cells (53 versus 47%) as compared with healthy individuals (75 versus 25%) (19). It is also known that in active SLE, a large expansion of CD27high plasmablasts/early plasma cells occur, reaching 20 times the amount found in healthy donors. These terminally differentiated CD19+/CD27high/slglow B cells display very low levels of FcγRII and CR1. All the control individuals showed also minimal levels of these receptors on their plasmablasts. A typical FACS profile of FcγRII and CR1 expression on plasmablasts from a healthy donor and an SLE patient is shown in Fig. 4. Major differences in the number of CD27high/slglow plasmablasts are emphasized by circles.

Discussion

It is well accepted that dysfunctions of FcγRs, complement and B-cell antigen receptors may contribute to the initiation and/or maintenance of IC-mediated autoimmune disorders.
Control of cognate interactions and inflammatory responses, IC clearance, B-cell selection and activation may all be affected by altered expression or function of these receptors. Various censoring mechanisms, including anergy and sequestration, prevent the participation of mature autoreactive B cells in germinal centre reactions, thereby avoiding their expansion into memory and plasma cells. Several studies suggest that IC-binding receptors may play a crucial role at checkpoints responsible for avoiding autoimmunity [reviewed in (20–22)].

Depending on the composition of IC and the degradation stage of complement components C3 and C4, IC-binding receptors may influence the fate of autoreactive B cells in distinct ways. FcγRIIb co-cross-linked with BCR regulates B-cell activation negatively (23). Similarly, human CR1 clustered by multimeric C3b on BCR-activated human B lymphocytes has also been shown to induce negative regulatory signals (17), a phenomenon demonstrated recently on human T cells, as well (24). On the other hand, CR2, which interacts with C3d, the final cleavage product of C3 has been described as a molecular adjuvant which lowers the threshold of B-cell activation by cross-linking CR2 to mIg (25).

In this study, we carried out a phenotypic analysis of the expression of IC-binding receptors on distinct B-cell subpopulations of healthy individuals and SLE patients. We found that the expression of the inhibitory receptors FcγRII

![Flow cytometric analysis of gated CD19+ B lymphocytes. Cells were triple stained with the following antibodies: FITC-labelled anti-CD21, PE-labelled anti-CD27 and Cy5-labelled anti-IgM F(ab')2, Ig isotype-matched FITC- and PE-conjugated mouse antibodies were used as negative controls for non-specific staining. (A) The expression of CR2 on IgM+ (dotted line) and IgM- (dashed line) memory B cells of a healthy control compared with naive cells (solid line). Histogram of one representative of eight independent experiments is shown. (B) The expression of CR2 on IgM+ (dotted line) and IgM- (dashed line) memory B cells of an SLE patient compared with naive cells (solid line). Histogram of one representative experiment of eight independent experiments is shown. (C) Average mean fluorescence intensity values of samples derived from eight healthy individuals and eight SLE patients treated as described above. Open columns, CD27− IgM+ cells; grey columns, CD27+ IgM+ cells; black columns, CD27+ IgM− cells of healthy individuals (C, control) and SLE patients, as indicated. Data are expressed as mean ± SE (Mann–Whitney U-test, P < 0.01).](image_url)

**Table 1.** sIg expression on CD27− naive and CD27+ memory B cells of control and SLE patients

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<th>sIgM expression (MFI)</th>
<th>CD27−</th>
<th>CD27+</th>
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<td>Control (n = 8)</td>
<td>338 ± 59&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1404 ± 139&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>SLE (n = 8)</td>
<td>767 ± 118&lt;sup&gt;b&lt;/sup&gt;</td>
<td>746 ± 71&lt;sup&gt;c&lt;/sup&gt;</td>
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<th>sIgG expression (MFI)</th>
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<tr>
<td>Control (n = 8)</td>
<td>—</td>
<td>1173 ± 99&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>SLE (n = 8)</td>
<td>—</td>
<td>689 ± 113&lt;sup&gt;d&lt;/sup&gt;</td>
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MFI, mean fluorescence intensity. Data are presented as mean ± SE. <sup>a,b,c</sup>P < 0.01, <sup>d</sup>P < 0.05.
and CR1 is up-regulated on memory B cells of healthy controls; however, their expression is considerably decreased on memory B cells of SLE patients. It is important to point out that, regarding the expression of CR2, the co-receptor for BCR, no difference was found between naive and memory cells of healthy individuals—in contrast to FcγRII and CR1. In SLE, however, a reduced expression of CR2 was characteristic for both the naive and memory cells. These findings further strengthen the notion that CR1 and CR2 expression is differentially regulated on human B lymphocytes under physiological conditions as well as in SLE. Taking into consideration that these two complement receptors have opposite effects on human B cells, their differential expression can be considered as an important regulatory component of B-cell function.

To get a further insight into which memory B-cell population is affected in SLE, we examined IgM⁺/CD27⁺ lymphocytes which participate in T cell-independent immune responses and class-switched memory cells produced in germinal centres after a T cell-dependent stimulus. We found that the reduced expression of FcγRs and complement receptors as well as sIg observed in SLE affected both IgM⁺ and switched memory B cells.

Memory B cells can be induced to differentiate into Ig-secreting plasmablasts in vitro upon stimulation (26). The checkpoints controlling the differentiation of self-reactive memory B cells into antibody-secreting plasma cells has yet to be determined, but it is feasible to suppose that abnormal expression of IC-binding receptors on anergic memory B cells may provide a signal sufficient to reactivate them. It is possible that under physiological conditions the inhibitory receptors FcγRII and CR1 serve as a final barrier to prevent these B cells from maturing into plasma cells, a function lacking in SLE patient.

It has been documented recently that in healthy individuals, the IgM⁺ memory B-cell compartment is depleted of self-reactive and polyreactive antibodies relative to the naive B-cell pool (27). Thus, transition from naive B cells into circulating IgM⁺ memory B cells is accompanied by efficient counter-selection against self-reactive naive B cells before the onset of somatic hypermutation. Based on this finding and along with the fact that IgM pathogenic auto-antibodies are less characteristic for SLE, we suppose that reduced levels of IC-binding receptors on IgM⁺ memory cells may contribute to autoimmunity in a way that is different from switched memory cells.

Fig. 4. FcγRII and CR1 expression on CD19⁺/CD27⁺/sIglow plasmablasts of healthy donors and SLE patients. Dot plot of gated CD19⁺ B cells, triple stained with the following antibodies: PE-labelled anti-CD27, Cy5-labelled anti-IgM F(ab')₂ and FITC-labelled anti-CD32 or FITC-labelled anti-CD35, as indicated. Ig isotype-matched FITC- and PE-conjugated mouse antibodies were used as negative controls for non-specific staining. The upper two dot plots show data obtained after staining for FcγRII on cells of a healthy individual and an SLE patient. The lower two dot plots show data obtained after staining for CR1 on cells of a healthy individual and an SLE patient. Major differences in the number of CD27⁺/sIglow plasmablasts are emphasized by circles. Data shown are representative of eight independent experiments.
Although little is known about the role of IgM+/CD27+ B cells in autoimmunity, disturbances in the memory cell compartment may result not only in higher susceptibility to infection with encapsulated bacteria but may also have other consequences. It has been shown recently that self-reactive antibodies including anti-nuclear and polyreactive antibodies were frequently expressed by IgG+ memory B cells in healthy donors (28). Under normal conditions, these cells remain in anergic state, but in situations where IC concentrations reach pathological levels, failed up-regulation of the inhibitory FcRII and CR1 on isotype-switched memory B cells may lead to activation and auto-antibody production.

In mice, there is accumulating evidence that the inhibitory FcγRII mediates its function during late stages of B-cell maturation (29). Absence of this receptor resulted in the expansion of IgG+ plasma cells secreting autoantibodies, but did not affect early events in the bone marrow such as receptor editing or did it prevent the development of IgM+ autoreactive B cells. Although both IgM+ and switched memory cells from SLE patients expressed lower levels of FcγRII, the functional consequences of this reduction may be different.

Mechanisms which are responsible for the reduced expression of IC-binding receptors in SLE may be due to both inherited and acquired elements. Whether complement containing IC acting as ligands or antibodies directed against these receptors are responsible for the reduction is unclear. An alternative way leading to reduced receptor density could be the proteolytic cleavage of extracellular domains of membrane proteins (ectodomain shedding). Several reports have proven that complement receptors CR1, CR2 (30, 31) as well as the inhibitory FcγRIlb from B cells (32) can be released and might be involved in regulatory processes. Terminal differentiation towards plasma cells may also account for the observed reduced receptor densities on B cells. Similarly to earlier reports (20), we also found a significantly higher percentage of CD19+/CD27+high/sIglow plasmablasts/early plasma cells and a disturbed ratio of naive and memory B cells in SLE patients. Investigating IC-binding receptors on these terminally differentiated CD27+high plasmablasts, we detected minimal levels of FcγRII and CR1 both in healthy individuals and SLE patients. Thus, the reduction in the expression level of these receptors on CD19+ B cells is not only due to disturbed memory compartment but also the higher frequency of CD19+/CD27+high plasmablasts expressing very low levels of these receptors.

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**Abbreviations**

- **BCR**: B cell receptor
- **CR1**: complement receptor type 1
- **CR2**: complement receptor type 2
- **FcγRII**: FcγR type II
- **IC**: immune complex
- **sIg**: surface Ig
- **SLE**: systemic lupus erythematosus

**References**


